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# Analysis of homeodomain function by structure-based design of a transcription factor

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**ABSTRACT** **The homeodomain is a 60-amino acid module which mediates critical protein–DNA and protein–protein interactions for a large family of regulatory proteins. We have used structure-based design to analyze the ability of the Oct-1 homeodomain to nucleate an enhancer complex. The Oct-1 protein regulates herpes simplex virus (HSV) gene expression by participating in the formation of a multiprotein complex (C1 complex) which regulates α (immediate early) genes. We recently described the design of ZFHD1, a chimeric transcription factor containing zinc fingers 1 and 2 of Zif268, a four-residue linker, and the Oct-1 homeodomain. In the presence of α-transinduction factor and C1 factor, ZFHD1 efficiently nucleates formation of the C1 complex *in vitro* and specifically activates gene expression *in vivo*. The sequence specificity of ZFHD1 recruits C1 complex formation to an enhancer element which is not efficiently recognized by Oct-1. ZFHD1 function depends on the recognition of the Oct-1 homeodomain surface. These results prove that the Oct-1 homeodomain mediates all the protein–protein interactions that are required to efficiently recruit α-transinduction factor and C1 factor into a C1 complex. The structure-based design of transcription factors should provide valuable tools for dissecting the interactions of DNA-bound domains in other regulatory circuits.**

Homeodomain proteins play central roles in the development and differentiation of eukaryotic organisms, but a mechanistic understanding of their biological specificity has only been achieved in a few cases (1, 2). Like other transcription factors, homeodomain proteins are modular, containing functionally and structurally independent domains which determine their sequence specificity and regulatory action (activation or repression). The DNA-binding activity of these proteins relies upon the 60-amino acid homeodomain, which has been extensively characterized biochemically and structurally (3–7). In several studies, functional differences between two regulatory proteins have been mapped to the homeodomains, even though the DNA-binding properties may be indistinguishable (8–13). These observations have suggested that the homeodomain serves not only as a DNA-binding module but also as a target for protein–protein interactions with other factors that enhance its target specificity and effector function (14–17).

The role of the human Oct-1 protein in the regulation of herpes simplex virus (HSV) gene expression exemplifies how protein–protein interactions with the homeodomain can determine functional specificity. Viral α or immediate early (α/IE) gene expression is controlled by the assembly of a multiprotein complex (C1 complex) composed of Oct-1, the viral α-transinduction factor (αTIF) protein (VP16, Vmw65, ICP25), and the cellular C1 factor (HCF), on the α/IE element (5′-ATGCTAATGATATTCTTTGG-3′) (18–26). The 5′ portion of the element is recognized by the Oct-1 POU domain, which is a bipartite DNA-binding domain. The POU-specific domain binds the ATGC subsite, and the associated homeodomain binds the TAATGA subsite (7, 27, 28). The 3′ portion of the element is recognized by αTIF and possibly by the C1 factor (27). Oct-2 has identical DNA-binding specificity to Oct-1 but forms the C1 complex with an efficiency lower than that of Oct-1 by a factor of 100 (22, 23). This difference between Oct-1 and Oct-2 in the regulation of HSV correlates with a single amino acid difference on the surface of their homeodomains which allows αTIF to distinguish between them in complex assembly (14, 15). The selective recognition of the Oct-1 homeodomain surface by αTIF exemplifies how protein–protein interactions can confer dramatically different abilities to regulate a particular gene upon two homeodomain proteins with identical DNA-binding specificity.

Previous studies established that the POU domain, composed of the POU-specific domain, a 24-residue linker, and the homeodomain, was as efficient as the full-length Oct-1 protein at binding the HSV α/IE element and forming the C1 complex (22). The isolated Oct-1 homeodomain was capable of nucleating complex formation but only functioned at high concentrations (14, 27). The low affinity and modest specificity of the homeodomain for DNA precluded an *in vivo* assay of C1 complex formation in the absence of the POU-specific domain.

We have used structure-based design to develop a chimeric protein, ZFHD1 (29), that has allowed us to test whether the homeodomain of Oct-1 can efficiently mediate all of the protein–protein interactions necessary to recruit αTIF and the C1 factor into a functional enhancer complex. ZFHD1 contains the Oct-1 homeodomain fused to two zinc-finger domains. The chimeric protein possesses high affinity and specificity for a hybrid DNA site, permitting the study of homeodomain function in the absence of the POU-specific domain and free of competition *in vivo* with the endogenous Oct-1 protein.

## MATERIALS AND METHODS

**Gel Electrophoretic Mobility-Shift Assays.** DNA-binding reactions contained 0.4–0.8 ng of DNA probe, 75 ng of sonicated salmon sperm DNA, 10 mM Hepes (pH 7.9), 0.5 mM EDTA, 50 mM KCl, 0.75 mM dithiothreitol, 4% (wt/vol) Ficoll 400, 300 μg of bovine serum albumin per ml, and the appropriate purified proteins or chromatographic fraction in a total volume of 10 μl. Reaction mixtures were incubated at 30°C for 30 min and resolved in nondenaturing 4% polyacrylamide gels (14). Protein–DNA complexes and the free DNA were quantitated by using a Molecular Dynamics PhosphorImager with IMAGEQUANT 3.22 software.

**Production of Recombinant Proteins.** The design and characterization of the glutathione *S*-transferase (GST)–ZFHD1 fusion has been described (29). GST–ZFHD1 variants con-

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Abbreviations: α/IE, α or immediate early; αTIF, α-transinduction factor; HSV, herpes simplex virus; GST, glutathione *S*-transferase.

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taining point mutations on the surface of the homeodomain were generated by PCR amplification of appropriate DNA fragments by using the expression vectors described below as substrates. The amplified fragments were then inserted into the *Bam*HI restriction site of pGEX-2T (Pharmacia) to generate in-frame fusions to GST. GST–ZFHD1 variants were expressed and purified as described (29).

**Transient Transfection Assays.** Reporter vectors were constructed by inserting the following fragments into the *Xho* I and *Kpn* I restriction sites of pGL2-Promoter (Promega): α/IE, 5′-GGTACCATGCTAATGATATTCTTTGGCTGCAGATGCTAATGATATTCTTTGGCTCGAG-3′; α/IE-ZF, 5′-GGTACCGCCCTAGTAATGATATTCTTTGGCTGCAGCGCCCTAGTAATGATATTCTTTGGCTCGAG-3′; α/IE-ZF-3′MT, 5′-GGTACCGCCCTAGTAATGCTGTTCTTTGGCTGCAGCGCCCTAGTAATGCTGTTCTTTGGCTCGAG-3′. These reporters were subsequently digested with *Bgl* II and *Dsa* I, end-filled with the Klenow fragment of *Escherichia coli* DNA polymerase I, and religated to remove the promoter region upstream of the TATA box that contains the 21-bp repeat elements of the simian virus 40 early promoter (30). The ZFHD1 expression vector was constructed by inserting a DNA fragment encoding the hemagglutinin epitope MYPYDVPDYA (31) and ZFHD1 (29) into the *Not* I and *Apa* I restriction sites of pRc/CMV (Invitrogen). Vectors expressing variants of ZFHD1 that contained mutations on the homeodomain surface were constructed as follows: The ZFHD1-encoding fragment was inserted into the *Not* I and *Apa* I restriction sites of pBluescript II SK(+) (Stratagene), and single-stranded DNA was produced to be used as the substrate for oligonucleotide-directed mutagenesis (32) using VCSM13 helper phage (Stratagene) according to the manufacturer's protocol. Mutagenesis was performed as described (32), except that T7 DNA polymerase was used in the initial polymerization step (33). Oligonucleotides used for mutagenesis contained ≈10 nt on each side of the mutation-specific bases. Products of the mutagenesis reactions were screened by dideoxynucleotide sequencing, and the desired fragments were isolated and inserted into the *Not* I and *Apa* I sites of pRc/CMV. The αTIF expression vector, pCMV1TIF1, containing the αTIF gene under control of the cytomegalovirus promoter was a gift from J. L. C. McKnight (University of Pittsburgh). The 293 cells were transfected and the results were quantitated as described (29).

## RESULTS

Recently we described the design and characterization of ZFHD1, a transcription factor composed of zinc fingers 1 and 2 from Zif268 fused to the Oct-1 homeodomain with a four-residue linker (29). ZFHD1 displayed DNA-binding specificity *in vitro* that was distinct from that of either parental protein and, when fused to an acidic activation domain, activated transcription in a sequence-specific manner *in vivo*. Although the optimal ZFHD1 binding site (29) has a different arrangement, computer modeling suggested that the linker between finger 2 and the homeodomain might also permit the ZFHD1 protein to bind DNA tightly in a configuration that would allow the homeodomain to be accessible for nucleation of a C1 complex. In this arrangement, finger 1 would bind the CNC triplet (CNCCCNNNTAATNN), finger 2 the CCN triplet (CNCCCNNNTAATNN), and the homeodomain would recognize the TAATNN sequence (CNCCCNNNTAATNN) (Fig. 1). This putative arrangement was used to design the α/IE-ZF element (5′-CGCCCTAGTAATGATATTCTTTGG-3′; Fig. 1), which represents a fusion of this putative ZFHD1 binding site with the 3′ portion of the α/IE element.

The Oct-1 POU domain and ZFHD1 were tested for binding to the α/IE and α/IE-ZF elements and for the ability to nucleate C1 complex formation (Fig. 2). As expected, the POU domain bound the α/IE element efficiently and readily formed the C1 complex upon addition of the αTIF and C1 factor (Fig. 2, lanes 1 and 2). In contrast, the POU domain had a significantly lower affinity for the α/IE-ZF element and did not efficiently nucleate the C1 complex on this site (Fig. 2, lanes 3 and 4). At high protein concentrations, the extent of POU domain binding to the α/IE-ZF element was comparable to that observed on the α/IE element; however, C1 complex formation was significantly less efficient on the α/IE-ZF element (Fig. 2, lanes 9–12). This probably reflects binding of the POU-specific domain to sequences in the 3′ portion of the α/IE-ZF element, analogous to that observed for a related α/IE element which also lacks an ATGC-binding site (35). Binding of the POU-specific domain to 3′ sequences would be

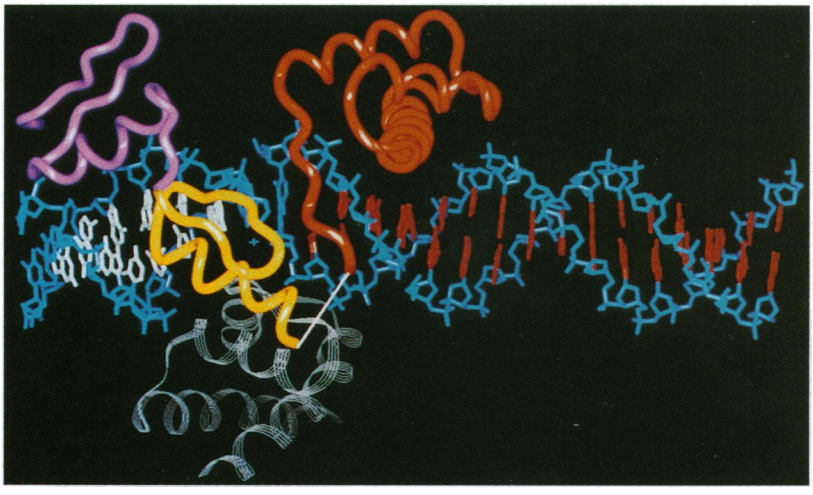

FIG. 1. Model of ZFHD1 bound to the α/IE-ZF element. Finger 1 of Zif268 is depicted in purple, finger 2 in yellow, and the Oct-1 homeodomain in red. The DNA in blue represents the α/IE-ZF element used in this study (5′-CGCCCTAGTAATGATATTCTTTGG-3′). The base pairs in the zinc-finger subsite (CGCCCT) are highlighted in white. The red base pairs represent the portion of the element (TAATGATATTCTTTGG) which includes the Oct-1 homeodomain core binding site (TAAT) and the 3′ sequences required for αTIF and C1 factor association. For reference, the Oct-1 POU-specific domain (gray) is shown at the position it would occupy when Oct-1 binds the wild-type α/IE element (5′-ATGCTAATGATATTCTTTGG-3′; POU-specific domain subsite underlined). This model was obtained by juxtaposing appropriate segments of the Oct-1 POU domain–DNA complex (7), the Zif268–DNA complex (34), and a model of B-form DNA used to represent the 3′ flanking sequences. The white line indicates the position of the linker that connects the C-terminal end of finger 2 and the N-terminal end of the homeodomain. This figure was generated with INSIGHT II (Biosym Technologies).

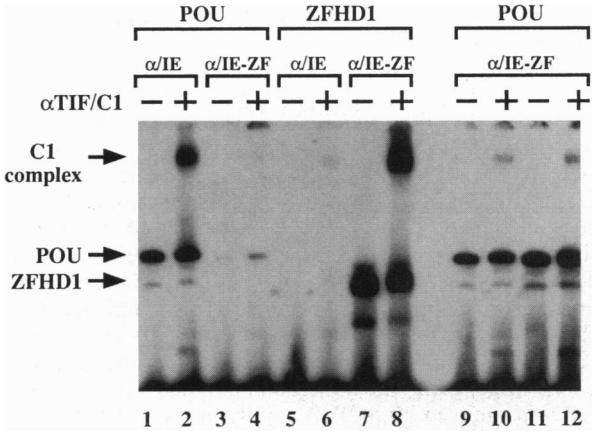


FIG. 2. Comparison of the POU domain and ZFHD1 for DNA-binding specificity and ability to participate in C1 complex formation. DNA–protein binding reactions were done as described in *Materials and Methods* with a probe containing either the α/IE (5′-GTGCATGCTAATGATATTCTTTGG-3′) [HSVα0 probe (22)] or the α/IE-ZF (5′-CGCCCTAGTAATGATATTCTTTGG-3′) element as indicated. The protein A–Oct-1 POU fusion protein (200 pg, lanes 1–4; 500 pg, lanes 9 and 10; 1000 pg, lanes 11 and 12) or the GST–ZFHD1 fusion protein (200 pg) were incubated in the absence (−) or presence (+) of 15 ng of protein A–αTIF and 1 μl of a chromatographic fraction containing the HeLa cell C1 factor (14), as indicated. The reactions contained subsaturating concentrations of αTIF and C1 factor. The position of the multiprotein C1 complex, as well as those of the POU–DNA and ZFHD1–DNA complexes, is indicated with an arrow.

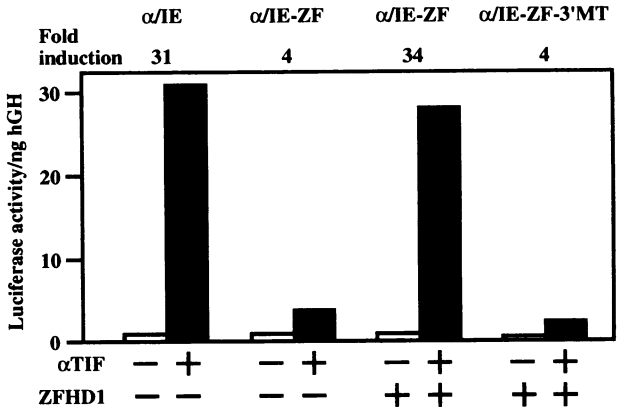


FIG. 3. Transcriptional activity of αTIF and ZFHD1 *in vivo*. The 293 cells were cotransfected with 5 μg of reporter vector, 10.05 μg total of expression vector (see below), and 5 μg of pCMV-hGH (hGH, human growth hormone) used as an internal control. Where indicated, cells were cotransfected with 10 μg of a vector expressing ZFHD1 (+) or the equivalent amount of the Rc/CMV vector alone (−) with (+) or without (−) 50 ng of pCMV1TIF1 (αTIF). Reporter vectors contained two tandem copies of the α/IE element (5′-ATGCTAATGATATTCTTTGG-3′), the α/IE-ZF element (5′-CGCCCTAGTAATGATATTCTTTGG-3′), or the α/IE-ZF-3′MT element (5′-CGCCCTAGTAATGCTGTTCTTTGG-3′). The amount of luciferase activity obtained, normalized to hGH production, was set to 1.0 for the cotransfection of pRc/CMV with the α/IE reporter. Each bar represents the average of three independent experiments. Actual values and standard deviation, reading from left to right, are as follows: 1.00 ± 0.26, 31.00 ± 4.17, 0.97 ± 0.26, 3.97 ± 0.46, 0.83 ± 0.03, 28.17 ± 4.53, 0.57 ± 0.07, and 2.37 ± 0.26. Fold induction refers to the ratio of normalized activity obtained in the presence of αTIF expression to that obtained in the absence of αTIF expression.

expected to sterically interfere with αTIF and C1 factor association.

ZFHD1 displayed a clear preference for the α/IE-ZF element. The designed factor did not bind the natural α/IE element or nucleate complex formation at this site (Fig. 2, lanes 5 and 6), but ZFHD1 efficiently bound the α/IE-ZF element and, upon addition of αTIF and C1, efficiently nucleated C1 complex formation (Fig. 2, lanes 7 and 8). Most important, comparable concentrations of the POU domain and ZFHD1 generated comparable DNA-binding activity and similar levels of C1 complex formation at their preferred sites. This argues strongly that the 60 amino acids in the homeodomain provide all the Oct-1-mediated protein–protein interactions necessary for efficient C1 complex formation. Furthermore, the results clearly indicate that the designed chimeric protein could be used to recruit the enhancer complex to a site which is not efficiently recognized by the wild-type Oct-1 protein.

To determine whether ZFHD1 could target enhancer complex formation *in vivo* to the α/IE-ZF element, transient transfection experiments were performed with 293 cells (Fig. 3). Cells were cotransfected with a vector expressing αTIF and a reporter construct containing two tandem copies of either the α/IE or the α/IE-ZF element upstream of a minimal promoter and the luciferase gene (see *Materials and Methods*). Cotransfection of cells with the αTIF expression vector and the α/IE reporter resulted in a 31-fold activation, reflecting assembly of the C1 complex with the endogenous Oct-1 and C1 factors. Both of these cellular factors are quite abundant and are probably not limiting for activity. In contrast, cotransfection of cells with αTIF and the α/IE-ZF reporter resulted in only a 4-fold activation, presumably reflecting the reduced ability of the endogenous Oct-1 to bind to the α/IE-ZF element and form a C1 complex. Cotransfection of cells with vectors expressing ZFHD1 and αTIF, together with the α/IE-ZF reporter, resulted in a 34-fold activation. Further controls confirmed that activation was a consequence of αTIF association and C1 complex assembly. A third reporter contained a mutant α/IE-ZF element, designed to support ZFHD1 binding but containing nucleotide substitutions (22) expected to abrogate αTIF binding and complex assembly (α/IE-ZF-3′MT; CGCCCTAGTAATGCTGTTCTTT). Cotransfection of cells with αTIF and ZFHD1, together with this reporter, resulted in only a 4-fold activation. These results demonstrate the affinity and specificity of ZFHD1 for the α/IE-ZF element and the ability of ZFHD1 to recruit αTIF and C1 factors *in vivo*.

The ability of ZFHD1 to nucleate C1 complex formation and activate transcription at the α/IE-ZF element should be dependent on the recognition of the Oct-1 homeodomain surface that is solvent exposed when the domain binds DNA (14, 15). Several mutations on the surfaces of helices 1 and 2 of the homeodomain have been previously characterized *in vitro* for their effect on the cooperative interaction with αTIF and on C1 complex assembly (14). Five of these mutations were individually introduced into ZFHD1 and then assayed, *in vivo* and *in vitro*, for complex formation on the α/IE-ZF element. Single amino acid substitutions at positions 18, 22, and 30 on the homeodomain surface have been shown to severely reduce the ability of Oct-1 to participate in complex assembly, while substitutions at positions 11 and 39 have milder effects. As expected, these mutations had similar effects in the context of the chimeric ZFHD1 protein without affecting the DNA-binding characteristics of the designed protein (Fig. 4*A* and *B*), confirming that the arrangement of the homeodomain in the ZFHD1/DNA complex mimics its arrangement in the POU domain/DNA complex. When compared with the 34-fold activation observed with the wild-type ZFHD1, the activation observed with the variants was 18-fold (N11A), 4-fold (K18E), 4-fold (E22A), 4-fold (E30Q), and 30-fold (N39H) (Fig. 4*A*). The extent of *in vitro* complex formation for the variants directly paralleled their activity *in vivo* (Fig. 4*B*). When compared with the level observed with the wild-type ZFHD1, the extent of C1 complex formed was 65% (N11A), 4% (K18E), 4% (E22A), undetectable (E30Q), and 102% (N39H)

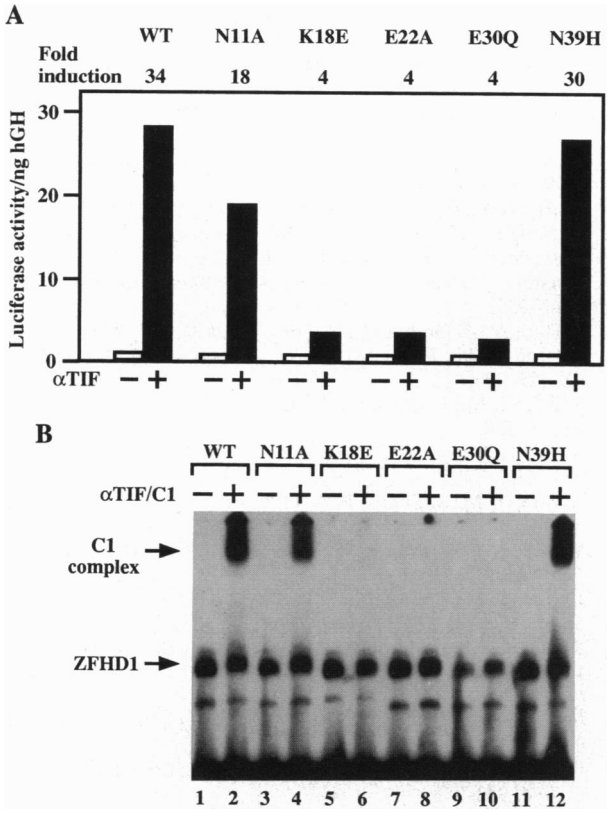


FIG. 4. ZFHD1-mediated C1 complex formation depends upon recognition of the homeodomain surface. (*A*) The 293 cells were cotransfected with 5 μg of reporter vector, 10.05 μg total of expression vector (see below), and 5 μg of pCMV-hGH (hGH, human growth hormone) used as an internal control. Cells were cotransfected with 10 μg of ZFHD1 expression vector encoding wild-type ZFHD1 (WT) or the indicated variants, together with the reporter containing the α/IE-ZF elements with (+) or without (−) 50 ng of pCMV1TIF1 (αTIF). Luciferase activity was normalized described as in the legend to Fig. 2. Each bar represents the average of three independent experiments. Actual values and standard deviations, reading from left to right, are as follows: 0.83 ± 0.03, 28.17 ± 4.53, 1.03 ± 0.30, 18.73 ± 2.63, 0.77 ± 0.33, 3.30 ± 0.93, 0.80 ± 0.23, 3.23 ± 0.57, 0.73 ± 0.26, 2.63 ± 0.28, 0.90 ± 0.15, and 26.83 ± 3.10. Fold induction refers to the ratio of normalized activity obtained in the presence of αTIF expression to that obtained in the absence of αTIF expression. Comparable levels of expression of all ZFHD1 variants were confirmed by the presence of a gel-shift activity, present only in extracts of transfected cells, that was reactive to the anti-hemagglutinin monoclonal antibody 12CA5 (gift from K. Moberg, Massachusetts Institute of Technology). (*B*) The GST–ZFHD1 fusion protein (200 pg) or variants containing the indicated substitutions were incubated in DNA–protein binding reaction mixtures in the absence (−) or presence (+) of 15 ng of protein A–αTIF and 1 μl of a chromatographic fraction containing the HeLa cell C1 factor. The probe contained the α/IE-ZF (5′-CGCCCTAGTAATGATATTCTTTGG-3′) element, and the reaction mixtures contained subsaturating concentrations of αTIF and C1 factor. The positions of the multiprotein C1 complex and the ZFHD1–DNA complexes are indicated with arrows. WT, wild-type ZFHD1.

(Fig. 4*B*). The results confirm that recognition of the surface of the Oct-1 homeodomain is critical for the assembly of the C1 complex *in vivo*.

## DISCUSSION

Structure-based design can provide valuable tools for studying the biological functions of transcription factors. We have used this strategy to dissect the Oct-1 POU domain and examine the role of the homeodomain in HSV α/IE gene regulation. The chimeric ZFHD1 protein nucleated the C1 complex on the α/IE-ZF element *in vitro* with an efficiency comparable to that of the intact POU domain on the α/IE element. This is the best evidence to date that the Oct-1 homeodomain alone provides all of the protein–protein interactions that Oct-1 contributes to C1 complex formation. The primary role of the POU-specific domain in the formation of the wild-type C1 complex is to enhance the affinity and specificity of homeodomain–DNA association. The POU-specific domain can be replaced by the two zinc fingers in ZFHD1 (Fig. 1) without compromising the ability to nucleate C1 complex formation.

Both Oct-1 and C1 factor are abundant nuclear proteins that are expressed in most, if not all, cell types. The use of the α/IE-ZF element and the designed ZFHD1 factor permitted the assay of C1 complex formation *in vivo*, independent of endogenous Oct-1 activity. ZFHD1 stimulated gene expression *in vivo* through the α/IE-ZF element to a level (34-fold) comparable to that observed at the α/IE element with the endogenous Oct-1 protein (31-fold). This activity was dependent on (*i*) cotransfection of ZFHD1 with αTIF, (*ii*) sequences 3′ to the ZFHD1 binding site in the α/IE-ZF element which are recognized by αTIF and possibly the C1 factor, and (*iii*) the appropriate amino acids, which are recognized by αTIF, on the surface of the homeodomain (14). This specificity indicates that of the 743 amino acids in the Oct-1 protein, the 60-amino acid homeodomain is sufficient to nucleate C1 complex formation *in vivo* when efficiently targeted to the appropriate DNA sequence.

The structure-based design of ZFHD1 has permitted this analysis because the unique DNA-binding specificity of the designed factor targets the homeodomain to a distinct DNA site without disrupting the homeodomain–DNA interaction. Related structure-based strategies should provide useful approaches to the study of gene regulation in many other systems. The use of ZFHD1 for the analysis of homeodomain function provides an example of how these design strategies can be used to characterize the biological activity of DNA-bound domains.

The chimeric ZFHD1 protein may also be useful for dissecting the role of the Oct-1 homeodomain in other regulatory contexts. Oct-1 is important for the regulated expression of the small nuclear RNA genes, which are ubiquitously expressed, the histone H2B gene, which is expressed in a cell-cycle-specific fashion, and the interleukin 2 and immunoglobulin genes, which are expressed only in lymphoid tissues (36). It is possible that regulatory specificity in some or all of these contexts will be determined by recognition of the homeodomain surface by cellular homologs of αTIF. Indeed, the unique activity of the Oct-1 binding site in B cells has recently been attributed to a B-cell specific factor, OCA-B (Bob1, OBF-1), which associates with the Oct-1 POU domain and contains an activation domain (37–39).

The recognition of the homeodomain surface mediates functional specificity in a number of other systems. In *Drosophila*, for example, the differing abilities of the Ultrabithorax (UBX) and Antennapedia homeodomain proteins to regulate the decapentaplegic gene are determined by their differing potentials for cooperative enhancer binding with the extradenticle (EXD) protein (16, 17). The interaction of UBX with EXD is dependent on residues 22, 24, and 56 on the UBX homeodomain surface (numbering scheme used in the homeodomain structural studies) and on the region C-terminal to the UBX homeodomain (16). Cooperative interaction with EXD also appears to modulate the target specificity of the abdominal-A and engrailed homeodomains (17). In human cells, the surfaces of helices 1 and 2 of the Phox1 homeodomain are important for the ability of the protein to recruit SRF and signal-responsive accessory factors to the *FOS* serum response element (40). These studies underscore the importance of being able to dissect the specific protein–DNA and protein–protein interactions that occur at the homeodomain surfaces. Because the structure and DNA docking of homeodomains are

so highly conserved (4–7), it may be possible to design ZFHD1 analogs with other homeodomains.

The study of transcription factor function has benefited greatly from the construction of chimeric proteins. For example, investigations of activation domains were greatly facilitated by fusing them to heterologous DNA-binding domains which would target the effector domain to an alternative DNA site that would not support the binding of competing activities (41, 42). The success of these studies depended on the ability of activation domains to function when presented in a variety of spatial contexts. For the targeting of a DNA-binding domain to an alternative site, one must ensure that the chimeric protein permits the specific orientation that is required for DNA binding. Structure-based design provides a powerful technique for retargeting individual DNA-binding domains in a defined spatial arrangement.

An attractive feature of the structure-based design strategy is that it offers the opportunity to change binding specificity without introducing any mutations at the protein–DNA interface. For some domains, such mutations may be difficult to obtain (43) or may inadvertently influence the interaction with other factors (44–46). Computer modeling studies (29) suggest that the rational design of chimeric DNA-binding domains should be possible for many different DNA-binding modules, facilitating characterization of the protein–protein interactions that may define their biological activity.

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